Research Article

The protection of acute spinal cord injury by subarachnoid space injection of Danshen in animal models

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Context/Objective: Following acute spinal cord injury (ASCI) in rabbits, subarachnoid space injection of Danshen was performed to protect the neurological damage. In this study, we established rabbit models of spinal cord injury using a modified Allen's method.
Design: After the operation introducing the injuries, the rabbits were randomized into two different groups, control group (normal saline, NS) and Danshen, a component extracted from Chinese herb, treatment group. Each rabbit was supplied with either the drug or placebo at 0.3 ml/kg each day through subarachnoid cavity.
Setting: Rabbit model of acute spinal cord injury were used for the response to Danshen treatment.
Participants: Total 48 Chinese rabbits aged four~ five months old provided by Experimental Animal Center of Hubei Province were used for this study.
Interventions: Danshen drug or placebo was administered via a silicon tube embedded under the spinal dura mater to administer the drugs into subarachnoid cavity.
Outcome Measures: After the treatment, damage indicators including cell apoptosis, morphological changes and oxidative damages were assessed.
Results: We found out that cell apoptosis was decreased after Danshen injection as determined by downregulation of apoptosis index (AI) by TUNEL analysis as well as propidium iodide (PI) percentage by FACS analysis. In the meanwhile, we observed cells after the treatment have increased numbers of BCL-2 positive cells, this indicated the antiapoptotic gene expression is increased after Danshen treatment. When we check the oxidative damage indicators, we found superoxide dismutase (SOD) was increased and malondiadehyde (MDA) levels were decreased after the treatment.
Conclusion: Danshen can protect ASCI through inhibition of oxidative damage in the injured cells and thus reduce the subsequent cell apoptosis in the spinal.

Key words: acute spinal cord injury, Danshen, oxidative damage, spinal, rabbit model

Introduction

Acute spinal cord injury (ASCI) occurs following different types of crushes, and it will cause major clinical health problems.[1,2] It is a consequence of mechanical trauma, which will lead to spinal cord core area damage. After injury, it will result in motor and sensory impairment of the patient.[3,4] Currently there are various studies of investigating reduce the damage after ASCI using various drugs and compounds.[1,5–11] We sought to investigate the potential role of Danshen, a Chinese herb extract, in relieving the damage via subarachnoid space right after the damage, using rabbit as animal model.

It has been shown that direct or indirect physical trauma to the spinal cord will lead to neuronal apoptosis, interruption of nerve conduction, which result in decreased neuronal survival and axonal growth.[12–15] More importantly, a recent study shows that antioxidant could play a key role on reducing progressive tissue damage through mitochondria biogenesis, which would result in the improvement of recovery after SCI.[16] Mitochondria dysfunction in the neural system could also lead to some neurodegenerative diseases,

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including mitochondrial encephalo-myopathy, ataxia, neuropathy, and others.[17,18] To evaluate the damage on the spinal cord, we not only assessed the changes of cell proliferation and apoptosis at spinal cord in addition to morphological changes, but also changes of the lipid peroxidation changing via measuring of the malondialdehyde (MDA) level and oxidative stress via level of superoxide dismutase (SOD),[9,19,20] using a rabbit model with ASCI and treated with Danshen. This will provide us insight for the treatment of human patient suffering spinal cord injury.

Methods

Animals

Four~ five months old Chinese rabbits of weight between 2.0 and 2.5 kg were provided by Experimental Animal Center of Hubei Province. Total 48 rabbits were randomized into three different groups of control group, Danshen treated group and pseudo-surgery group. The rabbits were anesthetized with 3% phenobarbital via ear vein. Following this, a dorsal midline incision after sterile condition and spinal cord injury was established using modified Allen's method. In brief, T12~L1 spinous processes and lamina were excised to expose the spinal dura mater. A 10 g iron hammer was then allowed to fall from 12 cm height freely (10 g x 12cmf) onto the dural sac to cause damage. The rabbit exhibited the retraction of both hind limbs. This indicates the success of the modeling. After the damage, a 2 mm silicon tube was embedded under the spinal dura mater and incision is closed. The Danshen drug were administered via the tube at 0.3 ml/kg each day. For the control treatment group was administered with saline at 0.3 ml/kg each day. All experiments were approved by Institutional Animal Care and Use Committee.

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay

Six rabbits were anesthetized 72 hours after injury for each group. The chest was opened and aortic cannulation was carried out using saline via the left ventricle of the rabbit until liquid became clear. Following this, 4% paraformaldehyde in PBS was injected to fix the tissue for 30 minutes and the injured spinal cord was excised and sectioned at 5 μm thickness. The TUNEL assay was conducted according to the manual of TUNEL kit (Roche). Briefly, each section was washed with PBS and incubated with 20 μg/ml proteinase K at 37°C for 30 minutes for digestion. After that, the sections were washed with 1X PBS and incubated with TUNEL reaction mixture at 37°C for 60 minutes in dark. The sections were sealed and observed under light microscope. Those cells with brown color nuclus were determined as TUNAL positive cells. The photos were analyzed using HPIAS-1000 software to calculate the apoptosis index (AI) as percentage of TUNEL positive cells among total cells.

Apoptosis analysis

Six rabbits were anesthetized 72 hours after the injury from each group, and the injured spinal cord were excised from each animal. Freshly isolate single cell suspension were obtained and stained with propidium iodide (PI) to determine the apoptotic cells using flow cytometry analysis on FACSCalibur (BD). The percentage of apoptosis is determined by PI positive cells among total cells.

Immunohistochemistry (IHC) staining

Paraffin embedded sections of injured spinal cord were deparaffinized and stained with Bcl-2 IHC SABC kit (Boster). Briefly, the sections were first stained with Bcl-2 antibody, followed by the incubation of biotinylated secondary antibody. After incubation and wash with 1X PBS, streptavidin-peroxidase were incubated to the tissue section followed by DAB chromogenic substrate. The Bcl-2 positive cells were quantified as those brown cells of every mm2 under light microscope.

Morphological analysis of spinal cord

The morphological changes were assessed 24 hours after injury. Twenty-four hours after injury, 4 rabbits from each experimental group were anesthetized and the injured spinal cords were excised and fixed with 10% formalin, followed by H&E staining of the paraffin embedded tissue sections. The morphology changes of the cells were determined under the light microscope.

The excised injured spinal cords were also fixed by 3% glutaraldehyde for 7 days, washed with phosphate buffer, fixed with 10% osmium and dehydrated with acetone. The tissues then were embedded with epoxy resin and examined under electron microscope.

superoxide dismutase (SOD) and lipid peroxidation (MDA) analysis

The injured area tissue was removed 8 hours after injury and was homogenized in the cold assay buffer on ice. The tissue homogenates were centrifuged at 10,000x g for 15 minutes at 4°C. The supernatant was collected, and SOD levels were measured according the kit booklet protocol, which utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. And one unit of SOD is defined as the amount of enzyme needed to exhibit

50% dismutation of superoxide radical. MDA level was assessed with the thiobarbituric acid reactive substances TBARS (TCA method) assay kit according to the manual from the manufacture.

Statistical analysis

All the statistical analysis were performed using student's t test and shown as mean ± standard deviation. *P < 0.05, **P < 0.01.

Results

Morphological change of the cells by the treatment of Danshen after spinal cord injury

Comparing with normal spinal cord structure (Figure 1A), acute spinal cord injury will lead to morphological changes of the cells (Figure 1B), including bubbles in both mitochondria and plasma within the cell, aggregation of chromosome, as well as dissociation of myelin sheath. These are all signs of apoptosis and early stage of cell death. After Danshen treatment for 3 days, we observed restoration of cell morphology comparable to normal spinal cord, with slight expansion of endoplasmic reticulum (ER) as well as mild swollen of mitochondria (Figure 1C). In contrast to early apoptosis of glial cells (Figure 1D), treatment of Danshen can greatly relief the damage caused by spinal cord injury. This is further confirmed by H&E staining (Figure 1E & 1F).

Cell death and apoptosis changes by the Danshen treatment

We further evaluated the mechanism of how danshen rescued the tissue damage after spinal cord injury. We first evaluated cell apoptosis/cell death by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, a method for detecting apoptotic DNA fragmentation that exist in the apoptotic cells. This assay relies on the use of terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyzes attachment of deoxynucleotides. By labeling of 3'-hydroxyl termini of DNA double strand breaks that generated during apoptosis, this can detect cell apoptosis at early stage. We found that spinal cord injury shows significant amount of cell apoptosis (Figure 2A), which is consistent with what we observed by morphological changes of the cells (Figure 1). The treatment of Danshen can reduce the levels of cell apoptosis by 57% (Figure 2B, Table 1). Furthermore, we also evaluated the Bcl-2 levels after spinal cord injury with or without Danshen treatment (Figure 2C, 2D), which indicates the survival of the cells. Treatment of Danshen can increase the levels of BCL-2 by 42% (Table 1) at protein level as determined by IHC staining. Consistent with this, the apoptotic cells percentage is also down-regulated by Danshen treatment as determined by PI levels through flow cytometry analysis (Table 1).

Oxidative stress evaluation

It is reported previously that spinal cord injury would cause mitochondria dysfunction in the neural system, and antioxidant has shown be able to reduce progressive tissue damage through mitochondria biogenesis.[16] We sought to evaluate whether Danshen could rescue

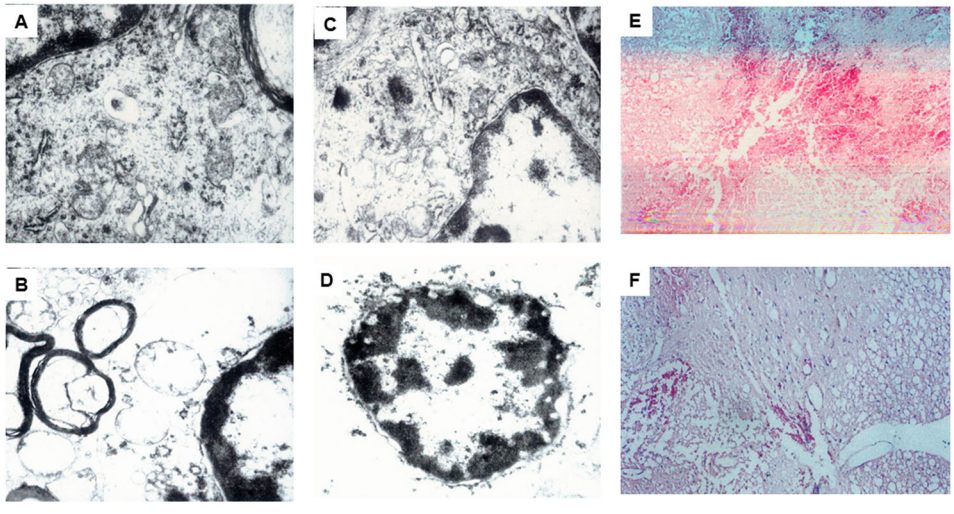

Figure 1 Morphological changes of spinal cord after ASCI. The morphological changes of cells within spinal cord were determined by electron microscope (x20,000). (A) Normal structure of spinal cord under electron microscope. (B) Control group of spinal cord 72 hours after injury and treated with saline. (C) Danshen treated group 72 hours after ASCI. (D) Early apoptosis of neuroglial cell. The morphological changes were assessed by H&E staining 24 hours after injury for both control group (E) and Danshen treated group (F).

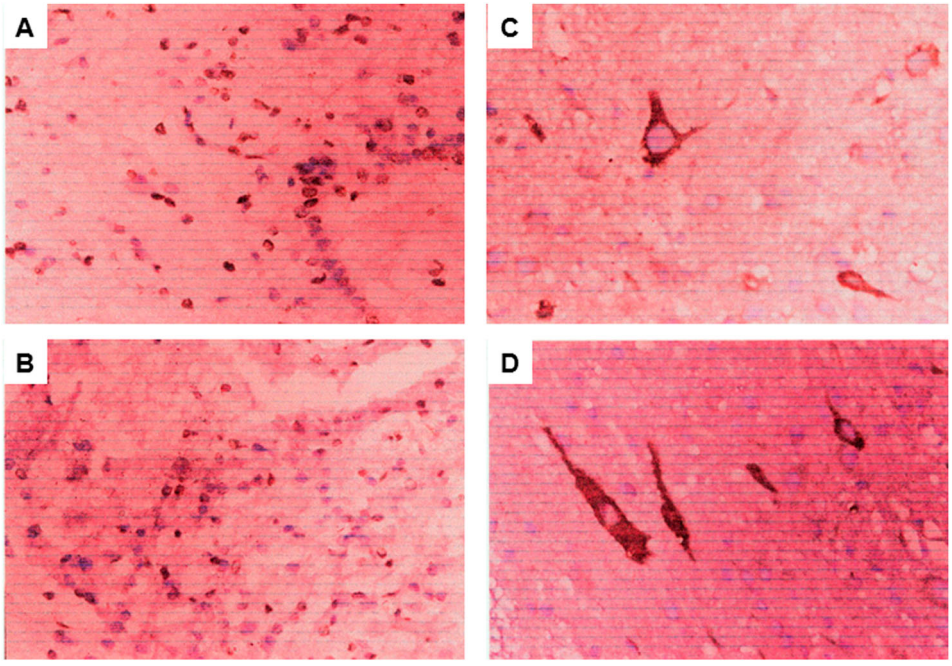

Figure 2 Cell death and apoptosis analysis by Danshen treatment after spinal cord injury. TUNEL analysis of control group (A) and Danshen treated group (B) 72 hours after injury. Bcl-2 analysis of control group (C) and Danshen treated group (D).

spinal cord injury through reducing oxidative stress. To achieve this goal, we evaluated levels of two products, malondialdehyde (MDA) for the lipid peroxidation changes and superoxide dismutase (SOD) for the oxidative stress in mitochondria. We found that after Danshen treatment, SOD level is increased for 33% comparing with control group with spinal cord injury (Table 2). On the other hand, MDA level is decreased for 50% after the treatment of Danshen.

Discussion

In this study, we used an animal model that mimic the acute spinal cord injury, to investigate the potential role of Danshen, a Chinese herb extract, on reversing the damage. We first examined the morphological changes of the cells in the spinal cord after the treatment of Danshen. We found that despite the hemorrhage and swollen in the spinal cord, especially within gray matter, accompanied by neuron cell death and nerve fiber dislocation, spinal cord from rabbits under the treatment of Danshen showed reduced hemorrhage and less nerve fiber dislocation and neuron cell death. This indicates treatment of Danshen could relieve the tissue damage after spinal cord injury, possibly through reduced cell death and apoptosis, as well as mitochondria biogenesis. Furthermore, Danshen can rescue tissue damage of spinal cord injury through reducing cell apoptosis and increasing cell survival ability, as determined by reduced apoptotic DNA fragmentation, decreased PI levels, and increased BCL-2 levels after the treatment.

To further evaluate the role of antioxidant on reducing progressive tissue damage through mitochondria biogenesis, we evaluated the impact of Danshen on both lipid peroxidation and oxidative stress. We found that after the treatment of Danshen, MDA level is decreased and SOD level is increased. This indicates that Danshen can reduce the tissue damage through reducing lipid peroxidation damage as determined by MDA levels, but not oxidative stress, which reflected by changes of SOD levels, at mitochondria that induced by spinal cord injury.

Table 1 Pathological analysis comparison after Danshen treatment.

Groups	TUNEL positive (AI)	Apoptosis (%)	BCL-2 (%)
control	30.39 ± 2.96	14.68 ± 2.81	13.37 ± 3.68
Danshen treatment	13.10 ± 1.38**	9.67 ± 1.09*	19.12 ± 4.74*

*P < 0.05, **P < 0.01.

Table 2 Injury related products quantification.

Groups	SOD (nu/ml)	MDA (mM)
control	101.70 ± 15.24	2.54 ± 0.69
Danshen treatment	136.20 ± 13.64**	1.27 ± 0.22**

**P < 0.01.

Conclusion

Our study provides insight of how Danshen could potentially protect patient suffering spinal cord injury, this is through reduced apoptosis, increased cell survival and down-regulated lipid peroxidation, but not through decreased oxidative stress. Further evaluation of combination of Danshen with other antioxidant against mitochondria dysfunction, might further improve the protection of spinal cord injury from oxidation induced tissue damage.

Disclaimer statements

Contributors None.

Ethics approval None.

Conflict of interest The authors declare there are no conflict of interest involved.

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